Simultaneous determination of metabolic stability and identification of buspirone metabolites using multiple column fast LC/TOF mass spectrometry

Application Note

Edgar Naegele

Abstract

A recent trend in the drug discovery and development process is to shift the starting point of drug metabolism and pharmacokinetic (DMPK) studies to a time as early as possible in the development chain to address potential issues in parallel with the optimization of the drug's lead structure. There is a strong need for assay methods to determine early ADME parameters such as metabolic stability and metabolite identification. It is necessary to work with a fast LC/MS system which provides the necessary functionalities together with high speed and resolution to handle the resulting large number of samples.

In this Application Note a metabolic stability profile of buspirone will be acquired by the Agilent 1200 Series Rapid Resolution LC/TOF system on a short Rapid Resolution High Throughput (RRHT) column. Simultaneously, the main metabolites will be identified in the same experiment by the automated use of a long RRHT column, which provides the necessary high resolution.

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Introduction

In modern pharmaceutical drug development it is of crucial importance to know the adsorption, distribution, metabolism and excretion (ADME) behavior of a new drug substance. The confident identification of metabolites derived from a drug substance even in very low concentrations is highly important because of the potential to cause a toxic reaction in humans. Therefore, a recent trend in the drug discovery and development process is to shift the starting point of drug metabolism and pharmacokinetic (DMPK) studies to a time as early as possible in the development chain to address potential issues in parallel with the optimization of the drug's lead structure[1].
To address this need, *in vitro* frontline assays are developed to identify potential problems in the development of a new drug substance. A typical experiment is the determination of the drug's metabolic stability and identification of the metabolites[4]. In this type of experiment the declining time-dependent parent drug concentration is monitored and the metabolites are identified from a highly metabolized sample at the end of the study[3].

It is necessary to work with a fast high throughput LC/MS system to handle the large number of samples generated by experiments where the metabolic reaction is stopped at various times in sample aliquots. The LC/MS system must be capable of analyzing samples in the single-digit minutes range. To achieve this goal, a system must be able to use multiple columns in a thermostatted column oven for various analytical methods and high throughput functionalities such as overlapped injections. State-of-the-art sub two micron particle columns are necessary to achieve high speed and resolution. It is possible to determine the metabolic stability semi-quantitatively and to identify the possible metabolites by empirical formula calculation from the measured highly accurate molecular masses together with an ESI time-of-flight mass spectrometer [4]. The connected time-of-flight mass spectrometer must be able to scan with the appropriate high speed for the fast LC system and to provide the necessary dynamic range to identify compounds existing in very low concentration with high sensitivity. In this Application Note a metabolic stability profile of buspirone will be acquired by the Agilent 1200 Series Rapid Resolution LC/TOF system on a short Rapid Resolution (RR) column. Simultaneously, the main metabolites will be identified in the same experiment by the automated use of a long RR column, which provides the necessary high resolution.

Equipment

Equipment (figure 1):

- Agilent 1200 Series binary pump SL with degasser. This pump has the capability to perform high resolving HPLC analysis on a 1.8-µm particle size column to get the best resolution and speed performance.
- Agilent 1200 Series high performance autosampler SL with thermostat. This autosampler is especially designed to work with the 1200 Series binary pump SL at the lowest delay volume.
- Agilent 1200 Series thermostatted column compartment (TCC). This TCC is ready for use with the Agilent 1200 Series binary pump SL with optional separate

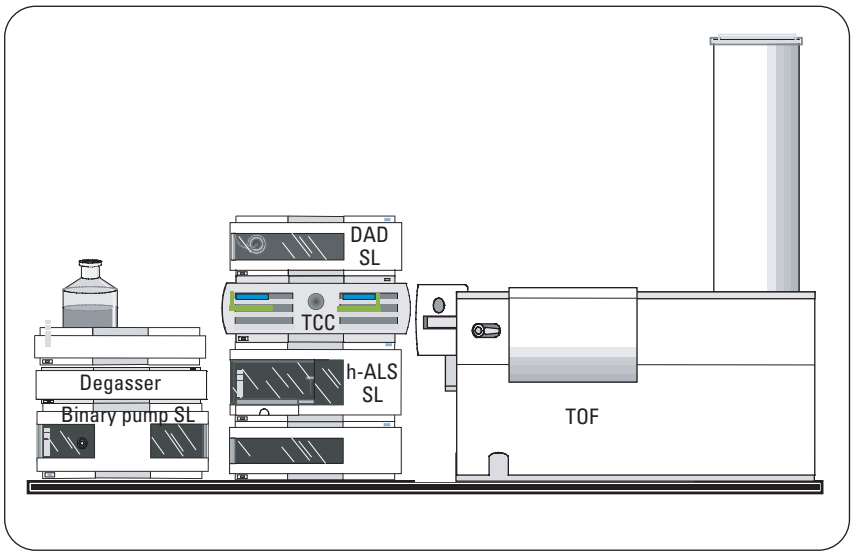

Figure 1
Instrument configuration for fast and high resolution LC/TOF with column switching. The TCC contains a 2-position/10-port valve and two low delay volume heat exchanger.

heat exchangers and post column cooling under optimized lowest delay volume conditions and alternating column use with a 2-position/10-port valve (figure 2).

- Agilent 1200 Series diode array detector SL (DAD). This DAD is capable of acquiring data at a sampling rate up to 80 Hz. In addition it has a built-in storage capability.
- Agilent 6210 Series TOF. Orthogonal acceleration time-of-flight mass spectrometer with dual sprayer interface for mass calibration to acquire molecular masses with highest accuracy. This time-of-flight mass spectrometer is capable of acquiring data at 40 Hz and utilizes pos/neg switching.
- Software: TOF software A02.00 for instrument control and Analyst software for data analysis.
- Columns:
  1) ZORBAX SB C18, 2.1 x 50 mm, 1.8-µm particle size
  2) ZORBAX SB C18 2.1 x 150 mm, 1.8-µm particle size.

The full performance of the Agilent 1200 Series binary system in the high resolution and fast LC configuration is demonstrated in two performance notes[5,6].

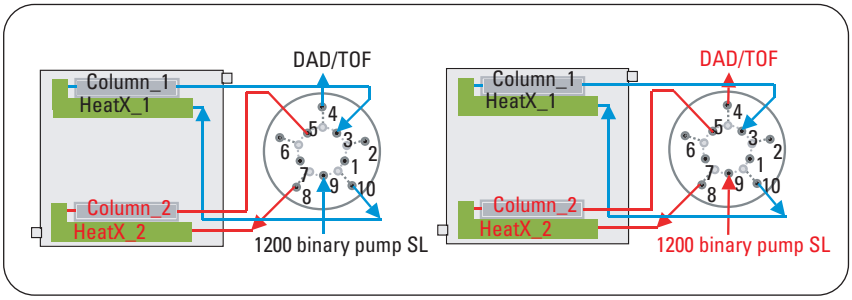

Figure 2
Column switching at the 2-position/10-port valve in the TCC for fast LC/TOF with column 1 [blue] (2.1 mm x 50 mm, 1.8-µm particle size) and high resolution LC/TOF with column 2 [red] (2.1 mm x 150 mm, 1.8-µm particle size).

Experimental

Sample preparation

The stock solutions contained:

- 20 mg/mL S9 preparation
- 0.1 mg/mL buspirone in water
- 1.6 mg NADP in 1,6 mL 0.1 M phosphate buffer pH 7.4
- 50 mM isocitrate/$MgCl_2$ (203 mg $MgCl_2$ x $6H_2O$ + 258.1 mg isocitrate in 20 mL H_2O)
- Isocitrate dehydrogenase (IDH) 0.33 U/mL

NADPH regeneration system:

- 1.6 mL NADP solution + 1.6 mL isocitrate solution + 100 µL IDH solution.

The incubation mixture consisted of:

- 3.85 µL substrate + 200 µL NADPH regeneration system + 746.15 µL phosphate buffer + 50 µL S9.

Incubation was carried out at 37 °C for 30 minutes and a 100-µL aliquot was taken every 5 minutes. The reaction was stopped by adding 100 µL perchloric acid and 6 µL acetonitrile followed by centrifugation for 15 min at 14,000 g. The supernatant was evaporated to dryness using a SpeedVac concentrator and reconstituted with water containing 0.1 % formic acid for LC/MS analysis as described below. For the control sample, incubation was stopped immediately at 0 min.

Methods

1) Fast LC method:
The Agilent 1200 Series binary pump SL was operated under the following conditions:

Solvent A: water + 0.025 % TFA;
Solvent B: ACN + 0.025 % TFA.
Flow rate: 1 mL/min
Gradient: 0 min 15 % B,
0.2 min 15 % B,
1.2 min 95 % B,
1.5 min 95 % B.
Stop time: 1.5 min
Post time: 2 min

The Agilent 1200 autosampler SL was used to make injections of 5 µL of sample with needle wash and the samples were cooled to 4 °C. Automated delay volume reduction and overlapped injection functions were used.
The DAD was used at 220 nm/ ref. 360 nm with the 2-µL cell.
The TCC was operated at 50 °C and column 1 was used.

2) High resolution LC method:
The Agilent 1200 Series binary pump SL was operated under the following conditions:

Solvent A: water + 0.025 % TFA;
Solvent B: ACN + 0.025 % TFA.
Flow rate: 0.5 mL/min

Gradient: 0 min 5 % B,
0.2 min 5 % B,
15 min 85 % B,
15.1 min 95 % B,
17 min 95 % B.
Stop time: 17 min
Post time: 10 min
The Agilent 1200 autosampler SL was used to make injections of 10 µL of sample with needle wash and the samples were cooled to 4 °C. Automated delay volume reduction was used.
The DAD was used at 220 nm/ Ref. 360 nm with the 2 µL cell.
The TCC was operated at 50 °C and column 2 was used.

3) The Agilent 6210 MSD TOF was used with the following acquisition parameters for both methods:
Source: ESI in positive mode with dual spray for reference mass solution.
Dry gas: 12.0 L/min.
Dry temp.: 350 °C.
Nebulizer: 50 psi.
Scan: 50-1000 at 40 Hz.
Fragmentor: 200 V or 300 V for CID.
Skimmer: 60 V
Capillary: 3000 V

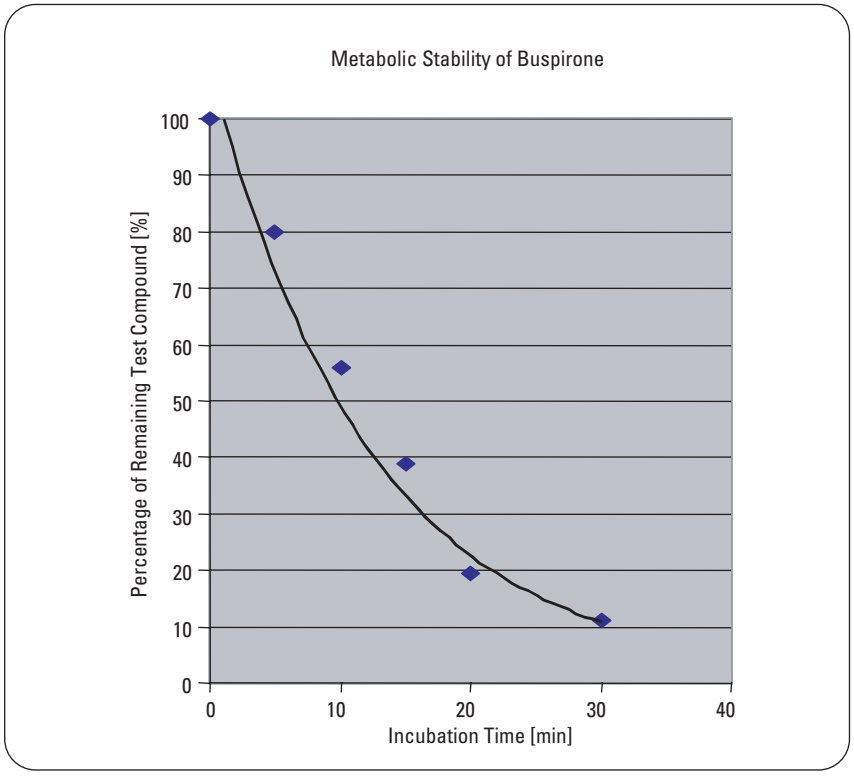

Figure 3
Determination of metabolic stability of buspirone by fast LC/TOF. After 20 minutes of incubation 80% of the buspirone is metabolized.

Results and discussion

Metabolic stability of buspirone

For the determination of metabolic stability a sample was taken from the incubation at time = 0, every 5 minutes up to 30 minutes and the metabolic reaction was stopped as described. The series of samples obtained was analyzed by fast LC/TOF on a 2.1 x 50 mm, 1.8-µm RRHT column within 1.5 minutes. The measured peak areas were compared to the peak area at t=0 of the control sample. The comparison showed that half of the buspirone was quickly metabolized in 10 minutes and about 80 % was metabolized in 20 minutes (figure 3). The concentration of the decreasing drug compound in the incubation mixture was monitored by ESI/TOF and the identity of the compound was controlled by means of accurate

mass measurement for the entire sample (figure 4). The mass accuracy values calculated for the empirical formula of buspirone $[M+H]^+$ ($C_{21}H_{32}N_5O_2$) = m/z 386.2556 was in the 1 ppm range in all instances. For example, the measured mass for the sample obtained after 30 minutes incubation time was m/z 386.2552 with a 1.04 ppm mass accuracy.

Identification of expected buspirone metabolites

For the identification of the expected metabolites, the highly metabolized sample that was obtained after 30 minutes incubation time was used. The sample was analyzed by high resolution LC/TOF on a 2.1 x 150 mm, 1.8-µm RRHT column with a 15-minute gradient. The expected metabolites with different oxidation reactions were extracted from the time-of-flight mass spectrum obtained. The identity of the metabolites was confirmed by means of accurate mass measurement using ESI/TOF and empirical formula calculation. For the mono oxidation reaction six different hydroxy buspirone metabolites $[M+H]^+$ ($C_{21}H_{32}N_5O_3$) = m/z 402.2505 were extracted and their molecular identity was confirmed by accurate mass measurement and empirical formula calculation with sufficient accuracy in the single digit ppm range (figure 5).

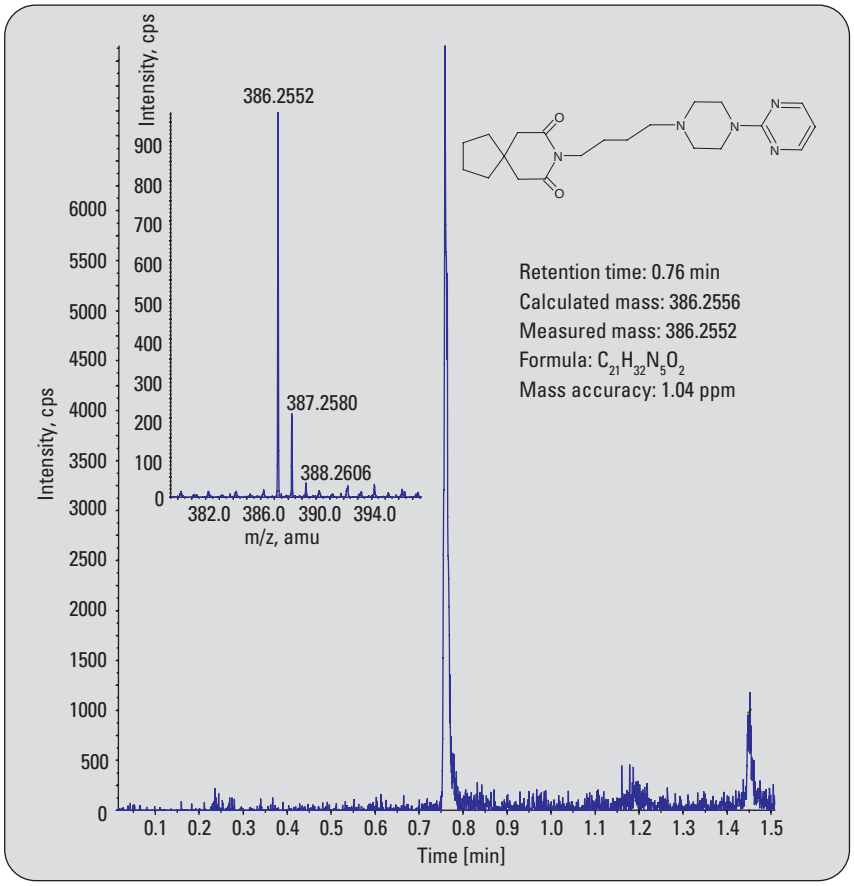

Figure 4
Measurement of accurate mass of buspirone from the metabolic stability incubation mixture at t=30 min by ESI/TOF and identity confirmation by empirical formula confirmation.

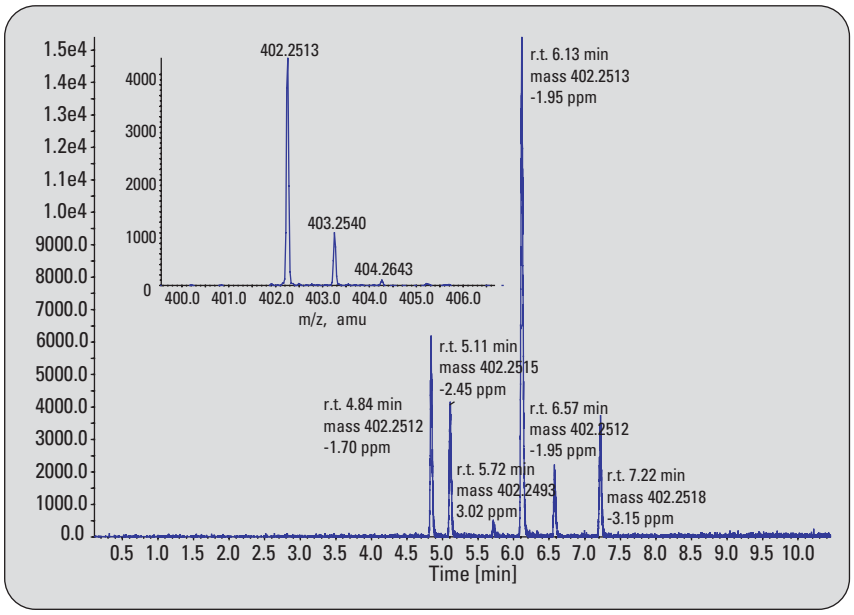

Figure 5
Hydroxy metabolites of buspirone $[M+H]^+$ ($C_{21}H_{32}N_5O_3$) = m/z 402.2505 were extracted from the MS TOF spectrum. Six metabolites were identified by accurate mass measurement and empirical formula confirmation.

A second set of identified expected metabolites are the doubly oxidized dihydroxy buspirone metabolites $[M+H]^+$ ($C_{21}H_{32}N_5O_4$) at m/z at 418.2454 (figure 6). The main component elutes at 5.74 minutes and the empirical formula was calculated from the measured mass with a high accuracy of -0.40 ppm.

Conclusion

An LC/MS method based on column switching with two 1.8-µm particle size columns for determination of metabolic stability and metabolite ID was developed. The short 50-mm column was used for the fast LC/TOF method to determine the metabolic stability in a short time by analyzing several samples at various time points. The long 150-mm column was used to achieve high resolution for the separation of a large number of metabolites. The method was tested with the drug substance buspirone. Applying the high resolution LC/TOF method on a 2.1 x 150 mm, 1.8-µm particle size column some expected metabolites were identified by empirical formula calculation with high mass accuracy.

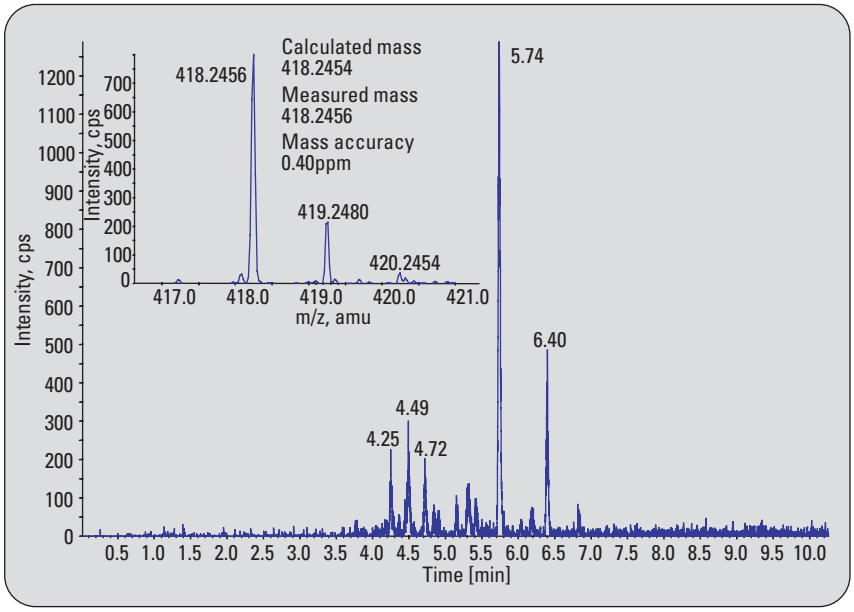

Figure 6
Dihydroxy metabolites of buspirone $[M+H]^+$ ($C_{21}H_{32}N_5O_4$) at m/z 418.2454 were extracted from the MS TOF spectrum. One main dihydroxy metabolite was identified by accurate mass measurement and empirical formula confirmation.

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Edgar Naegele is Application Chemist at Agilent Technologies, Waldbronn, Germany.

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